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G Protein control of inositol lipids in intact vascular smooth muscle

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Agonist-induced PIP_2 breakdown has been demonstrated in permeabilized vascular smooth muscle and shown to depend on a G protein. Segments of rat tail artery were permeabilized with ATP and EGTA after prelabeling with [^{3}H]inositol. Norepinephrine and GTPγS were both able to increase levels of IP, IP_2 and IP_3 in the segments. The effects of both norepinephrine and GTPγS on the segments was non-additive. Aluminum fluoride also increased inositol phosphates in intact segments and norepinephrine-stimulated increases in IP, IP_2 and IP_3 were insensitive to pertussis toxin.

Inositol lipid; Norepinephrine; Fluoride; GTPγS; Pertussis toxin; Rat tail artery

1. INTRODUCTION

Several hormones and neurotransmitters have been recently shown to stimulate the contraction of smooth muscle via the receptor-mediated breakdown of the lipid PIP_2 [1,2]. This lipid breaks down to form IP_3 and diacylglycerol [3]. The IP_3 then binds to its receptor on the sarcoplasmic reticulum and induces the release of calcium stored within the SR into the cytosol [4,5]. The diacylglycerol released can stimulate the activity of protein kinase C which can then phosphorylate a variety of cellular proteins [6]. The contraction of smooth muscle can be stimulated by the rapid rise in cytoplasmic calcium and perhaps by protein kinase C-stimulated protein phosphorylation as well [2,3].

In many different cellular systems G proteins have been shown to mediate the effects of agonists on the breakdown of PIP_2 [7–9]. The agonist binds to its receptor on the plasma membrane and stimulates the G protein to activate phospholipase C to hydrolyze the PIP_2. In these studies either GTP or GTPγS were shown to stimulate the hydrolysis of PIP_2 in either isolated membrane preparations or in permeable cell preparations [7–9]. Aluminium fluoride (AlF_4^-) was also shown to stimulate the breakdown of PIP_2 in many of these cells [10,11]. In most of the cell types studied the agonist-mediated breakdown of PIP_2 was not inhibited by pertussis toxin [12,13], although agonist-stimulated phospholipase C was very sensitive to pertussis toxin in a variety of tissues [14,15].

The purpose of the current study was to investigate the role of G proteins in intact vascular smooth muscle. We have found for the first time that GTPγS and norepinephrine can stimulate the breakdown of PIP_2 in permeabilized segments of rat tail artery. We have also found that AlF_4^- can stimulate PIP_2 breakdown in tail artery and that the norepinephrine-stimulated production of inositol phosphates in this tissue is insensitive to pertussis toxin. This indicates that a pertussis toxin-insensitive G protein most likely mediates the effects of norepinephrine on PIP_2 breakdown in intact vascular smooth muscle.

2. MATERIALS AND METHODS

2.1. *Intact arteries*

Rats were sacrificed by excess CO_2 exposure and the tail arteries quickly removed. The arteries were rinsed with bicarbonate-buffered PSS equilibrated with 95% O_2/5% CO_2 at 37°C and pH 7.4. The solution had the following composition (in mM): NaCl 116.7, KCl 4.5, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 22.5, NaH_2PO_4 1.2, Na_2SO_4 2.4, D-glucose 5.6. The arteries were dissected free of extraneous connective tissue and then cut with a scalpel into segments of 5 mm. The segments were incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 in a shaking water bath in 1 ml PSS for 60 min. The segments were then transferred to a tube containing 0.5 ml [^{3}H]inositol (10 μCi) in PSS and the incubation continued for 150 min. The segments were then treated with LiCl (10 mM) for 30 min and then treated with various agonists for 20 min before being quick-frozen in liquid N_2 and thawed in trichloracetic acid (10%, 4°C). The solutions were then extracted 4 times with ethyl ether to remove the trichloracetate. The solutions containing inositol phosphates were then applied to small (0.5 ml) columns of Dowex 1×8-400 (200–400 mesh) resin and eluted sequentially with 10 ml each of water, 0.2 M ammonium formate/0.1 M formic acid, 0.4 M ammonium formate/0.1 M formic acid, and 0.8 M ammonium formate/0.1 M formic acid. Each 10 ml step applied to each column was collected off the column as a single 10 ml fraction. Then 2.5 ml aliquots of each fraction were added to scintillation fluid (16 ml of Ecolume) and the

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Abbreviations: PIP_2, phosphatidylinositol 4,5 biphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); IP, inositol 4-monophosphate; IP_2, inositol 1,4-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; PSS, physiological saline solution; TES, *N*-tris[hydroxymethyl]methyl-2-amino-ethanesulfonic acid

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radioactivity in the samples determined with a Beckman LS3801 scintillation counter.

2.2. *Permeable arteries*

In order to measure the metabolism of inositol lipids in permeable rat tail arteries, the tail arteries were prepared, divided into segments, and pretreated with [^{3}H]inositol for 3 h as described for intact arteries. The arteries were then treated for 30 min at 20°C with permeabilization solution 1, using a modification of the procedure first applied to smooth muscle by Morgan and Morgan [16]. The composition of permeabilization solution 1 was as follows (in mM): EGTA 10, ATP 5, KCl 120, $MgCl_2$ 2, TES buffer 20, [^{3}H]inositol 0.025, 20 μCi/ml, CTP 0.1, pH 7.0. The arteries were then incubated for 30 min at 20°C with permeabilization solution 2. The composition of permeabilization solution 2 was as follows (in mM): EGTA 1, ATP 5, KCl 120, $MgCl_2$ 6, TES buffer 20, inositol 0.025, 20 μCi/ml, CTP 0.1, $CaCl_2$ 0.122, pH 7.0. The segments were then incubated at 37°C in permeabilization solution 2 together with LiCl (10 mM) and agonists. The incubations were terminated by the addition of 2 ml trichloracetate (10%, 4°C). The trichloracetate was removed with ether and then the acqueous extracts were applied to Dowex columns as described above.

2.3. *Materials*

Sprague-Dawley rats (male, 2–4 months old) were obtained from Taconic (Germantown, NY). Myo-[2-^{3}H]inositol was obtained from American Radiolabeled Chemicals (St. Louis, MO). Norepinephrine, Dowex 1×8-400, pertussis toxin, and GTPγS were obtained from Sigma (St. Louis, MO).

3. RESULTS

In preliminary experiments rat tail artery segments were prelabeled with [^{3}H]inositol, and then treated by the procedure of Morgan and Morgan [16] in order to first render them permeable to small molecules and then to subsequently reseal the membranes before treatment with norepinephrine. When these 'resealed' segments were then frozen and the inositol phosphates extracted and separated, it was found that the segments no longer contained any inositol phosphates. However, when we extracted the bathing medium outside the segments, a substantial amount of inositol phosphates were recovered. This indicated that the 'resealing' procedure, in our hands, did not succeed, but that norepinephrine could still stimulate the release of inositol phosphates from the permeable cells and these compounds could be measured in the medium outside the cells. Norepinephrine was shown to significantly stimulate the production of IP, IP_2 and IP_3 in permeable segments of rat tail artery (Table I). GTPγS (a non-hydrolyzable analogue of GTP) was also shown to significantly stimulate the production of the inositol phosphates in permeable segments of the tail artery. When segments of tail artery were exposed to both norepinephrine and GTPγS simultaneously, the production of inositol phosphates was increased in a non-additive process (Table I). This indicated that a G protein in the tail artery perhaps was capable of mediating the effects of norepinephrine on the phospholipase C that hydrolyzed PIP_2 in the tissue to form IP_3 and diacylglycerol.

Since other investigators have used a mixture of aluminum and fluoride ions to stimulate certain G proteins, we added AlF_4^- to tail artery segments that had been prelabeled with [^{3}H]inositol and found that the mixture was capable of sifnificantly stimulating the release of IP and IP_2 from the labeled lipid in the tissue (Table II).

Table II

Effect of norepinephrine and aluminum fluoride on inositol phosphates in rat tail artery

	cpm mean ± S.D. ($n=4$)	
	IP	IP_2
Control	3711 ± 240	778 ± 69
Norepinephrine	16576 ± 2349*	6326 ± 1047*
Aluminum fluoride	5223 ± 771**	1431 ± 86*

Rat tail artery segments were treated with [^{3}H]inositol as described in section 2 and then treated with either norepinephrine (10^{-5} M), $AlCl_3$ (10 μM) plus KF (5 mM) or nothing for 20 min and then frozen in liquid N_2 and thawed in trichloracetic acid (10%, 0°C). The inositol phosphates were separated by Dowex column chromatography as described in section 2.

Significantly different from control: *$P<0.005$, **$P<0.025$

Table I

Effect of norepinephrine and GTPγS on inositol phosphates in permeable tail artery

	cpm means ± S.D. (n=9)		
	IP	IP_2	IP_3
Control	1211 ± 248	257 ± 41	38 ± 3
Norepinephrine (10^{-5} M)	7504 ± 2782*	3088 ± 1634*	92 ± 30*
GTPγS (10^{-4} M)	2762 ± 607*	569 ± 144*	44 ± 5**
GTPγS + norepinephrine	4930 ± 2385*	1601 ± 756*	55 ± 12*

Segments of rat tail artery were treated with [^{3}H]inositol and permeabilized as described in section 2 before being treated with LiCl (10 mm) and the compounds listed above for 20 min at 37°C. The tubes were then extracted with 2 ml trichloracetate and the inositol phosphates in the samples separated by Dowex column chromatography as described in section 2.

Significantly different from control: *$P<0.005$, **$P<0.01$.

Table III

Effect of pertussis toxin on norepinephrine stimulation of inositol phosphate release in tail artery

	cpm m ± S.D. (n = 4)		
	IP	IP_2	IP_3
Control	244 ± 50	293 ± 38	316 ± 68
Norepinephrine (10^{-5} M)	11390 ± 2484	7171 ± 1187	753 ± 151
Pertussis toxin (5 μg/ml)	365 ± 145	418 ± 125	432 ± 50
Pertussis toxin + norepinephrine	7850 ± 1836	5263 ± 1271	864 ± 200

Segments of rat tail artery were preincubated with medium and with [^{3}H]inositol-containing medium as described in section 2. After 2 h in [^{3}H]inositol they were incubated for 1 h at 37°C with [^{3}H]inositol, LiCl (10 mM) either with or without pertussis toxin (5 μg/ml) and then treated with or without norepinephrine (10^{-5} M) for 20 min at 37°C before being frozen and extracted as described in section 2.

Pertussis toxin has been shown to inhibit the activity of certain G proteins in certain tissues, and so we added pertussis toxin to norepinephrine-stimulated segments of rat tail artery. Pertussis toxin did not significantly alter either the norepinephrine-stimulated levels of IP, IP_2, or IP_3 in the tissue or the background levels of the inositol phosphates observed in the segments of tail artery (Table III). It appeared that the G protein present in the smooth muscle of intact rat tail artery was insensitive to pertussis toxin.

4. DISCUSSION

Recently, Nishimura et al. [17], together with Kobayashi et al. [1] have shown that smooth muscle could be permeabilized with α-toxin and the resulting preparations would contract in response to agonists such as norepinephrine or carbachol only in the presence of GTP. This idicated that one or more G proteins most likely mediated the effects of these agonists on the contractions of the smooth muscle. Other investigators have demonstrated that either GTP or the non-hydrolyzable GTP analogue, GTPγS, could stimulate the breakdown of PIP_2 to form IP_3 in either cultured smooth muscle cell membranes [18] or in the homogenate of canine coronary arteries [19]. Similar studies have demonstrated that GTPγS could stimulate the breakdown of PIP_2 in either permeable cells or membranes from a variety of tissues [7,8]. These experiments indicate that one or more G proteins most likely mediate the action of a contractile agonist on phospholipase C directly. Further evidence in support of this theory comes from the work of those who have shown that the ion AlF_4^- can stimulate the breakdown of PIP_2 to form IP_3 in cells from ileum [20] and myometrium [12], as well as from other tissues [10]. Some of these studies have found that agonist-sensitive PIP_2 breakdown is inhibited by pertussis toxin [14,15] and pertussis toxin is believed to exert its effects on metabolism via the inactivation of G proteins. Other studies have found that agonist-sensitive PIP_2 breakdown is insensitive to pertussis toxin in several tissues [12,13]. Since PIP_2 breakdown in nearly all of the latter tissues is stimulated by either GTP, GTPγS, or by AlF_4^- it appears likely that a pertussis toxin-insensitive G protein is required to mediate the effects fo agonists on phospholipase C in such tissues.

In none of the above studies has anyone investigated the sensitivity of phospholipase C to GTP or GTP analogues in intact vascular smooth muscle, nor have they examined the sensitivity of this enzyme system to pertussis toxin in intact vascular smooth muscle. We have found that GTPγS can stimulate the breakdown of PIP_2 in permeabilized cells of rat tail artery, as does norepinephrine itself. The two agonists together do not exert additive effects on the production of inositol phosphates in this tissue. This indicates that GTPγS most likely interacts with the same G protein that norepinephrine must interact with in order to stimulate PIP_2 breakdown in the cells. We have also shown that inositol phosphate levels increase in rat tail artery in response to the addition of F^- and Al^{3+} salts, presumably resulting from the formation of AlF_4^- that many investigators have shown to stimulate the activity of G proteins. Since we have not found that agonist-mediated PIP_2 metabolism in rat tail artery is sensitive to pertussis toxin, one can conclude that the G protein involved is insensitive to this toxin. Of course, the incubation period employed in our experiments was short (60 min) and others have used much longer incubation periods (30 h [21]). However, we used a much higher concentration of pertussis toxin (5 μg/ml) than other investigators (10 ng/ml [15]), which might be expected to make up for the shortness of our incubation period. Our tissue would not survive 30-h incubation periods.

In conclusion, we have demonstrated that norepinephrine-stimulated PIP_2 breakdown in intact vascular smooth muscle requires the assistance of a pertussis toxin-insensitive G protein, and that this G protein no doubt plays a role in the agonist-stimulated contractions of this tissue.

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